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I Chromosomes during Spermatogenesis

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Little is known about the timing of meiotic prophase events during spermatogenesis in the mouse or how these events are related to cell-cycle progression. This work was designed to test hypotheses about the timing and biochemical correlates of developmental acquisition of competence to condense bivalent pairs of homologous chromosomes held together by chiasmata. The experimental approach takes advantage of the fact that okadaic acid (OA) treatment of pachytene spermatocytes causes precocious entry into metaphase I (MI) of meiosis. Leptotene and zygotene (L/Z) spermatocytes are not competent to respond to OA with condensation of chiasmate bivalent chromosomes. Competence for MI condensation of chiasmate bivalents is acquired by the middle of the pachytene stage of meiotic prophase, several days after homologous chromosomes become fully synapsed. The acquisition of MI competence is paralleled by the accumulation of histone H1t in the nuclei of mid-pachytene spermatocytes. Biochemical differences also exist between the incompetent L/Z spermatocytes and the competent pachytene spermatocytes. Both have the molecular components of metaphase promoting factor, CDC2 and CYCLIN B1; however, the histone H1 kinase activity of metaphase promoting factor of incompetent L/Z spermatocytes is not activated by OA, as it is in competent pachytene spermatocytes. Additionally, the CDC25C protein phosphatase is present in competent pachytene spermatocytes, but not in incompetent L/Z or early pachytene spermatocytes. Both incompetent and competent spermatocytes accumulate MPM-2 phosphoepitopes and phosphorylated histone H3 in response to OA treatment, indicating that presence of these antigens is not sufficient to promote condensation of meiotic chromosomes. These data demonstrate that meiotic competence of spermatocytes is acquired after homologous chromosome pairing is established and is coincident with first appearance of histone H1t and CDC25C protein phosphatase in spermatocytes.

Key Words: **meiosis; spermatogenesis; histone H1t; histone H3; CDC2; CYCLIN B1; MPF; CDC25C.**

INTRODUCTION

Condensation of metaphase I (MI) chromosomes is an essential prerequisite for normal chromosome segregation during spermatogenesis; however, it is not known what controls condensation of paired meiotic chromosomes nor how their condensation is related to genetic and cellular events of meiosis I prophase.

The cytological events leading to MI during meiosis in the male mouse are well characterized. During the first part of meiotic prophase, chromosomes pair and intimately synapse with their homologs; the hallmark of synapsis is the synaptonemal complex (SC), a proteinaceous structure that begins to assemble during the zygotene stage and is complete by the beginning of the pachytene stage. Chromosomes remain fully synapsed for approximately a week before making the transition out of the pachytene stage, marked by chromatin condensation through the diplotene and diakinesis stages to MI, when individual chromosomes are visible. During prophase, the molecular events of recombination occur within each homologous pair. The points of recombination, or chiasmata, are first visible during the diplotene stage, providing the first cytological evidence that recombination has occurred. Chiasmata hold the chromosomes together as they are oriented onto the spindle and thus ensure successful and accurate reductive segregation of chromosomes during the subsequent anaphase (Lamb *et al.*, 1997).

Although these cytological events are clear, very little is known about their relationship to molecular events of recombination. This is an important issue since recombi-

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nation is required to ensure accurate chromosome segregation. How does the meiotic prophase spermatocyte monitor the progress of meiotic recombination? How does it time the onset of the division phase so that it always occurs after, and not before or during, recombination? It is likely that the spermatocyte has an especially vigilant checkpoint to monitor completion of recombination before initiating chromosome condensation and progress to MI. In fact, proteins involved in control of cell-cycle progression (ATM, HSP70-2, CHK1) have been localized to the region of the SC, suggesting tight coupling of events at the SC and events signaling the end of meiotic prophase (Allen *et al.*, 1996; Flaggs *et al.*, 1997). However, the biochemistry of meiosis I prophase and the G2/M transition in spermatocytes is not well understood. The phenotypes of null mutations have indicated a requirement for a variety of specific proteins in order to complete meiotic prophase (Baker *et al.*, 1995; Dix *et al.*, 1996; Pittman *et al.*, 1998). Likewise, spermatocytes synthesize proteins known to govern cell-cycle transitions in other systems (Chapman and Wolgemuth, 1994; Wu and Wolgemuth, 1995). Nonetheless, there is a lack of understanding of the dynamic relationships among the biochemical mediators of recombination and cell-cycle progression as spermatocytes advance through meiotic prophase.

In all systems examined thus far, progression to mitotic or meiotic metaphase is controlled by the activity of metaphase-promoting factor (MPF), a complex of the cyclin-dependent kinase CDC2 ($p34^{cdc2}$) and the regulatory subunit CYCLIN B1. Activation of MPF results in the G2/M transition. It has not yet been demonstrated definitively that MPF controls the spermatogenic G2/MI transition, but it is likely that this is the case; MPF activity has been reported in pachytene spermatocytes (Chapman and Wolgemuth, 1994; Wiltshire *et al.*, 1995), and the high MPF activity of oocytes drives spermatocyte nuclei injected into oocytes to meiotic metaphase (Sasagawa *et al.*, 1998). MPF is activated by dephosphorylation of Thr-14 and Tyr-15 of CDC2 by the protein phosphatase CDC25C, which is also present in spermatogenic cells in mice and rats (Mizoguchi and Kim, 1997; Wu and Wolgemuth, 1995). However it is not known if CDC25C triggers the activation of MPF at the end of meiotic prophase in spermatocytes.

Experimental analysis of the dynamics of the progression of spermatocytes to meiotic metaphase has been facilitated by methods that allow short-term culture of pachytene spermatocytes (Handel *et al.*, 1995). Novel approaches emerged from the discovery that treatment of pachytene spermatocytes from adult mice with the phosphatase inhibitor okadaic acid (OA) induced rapid condensation of MI chromosomes (Wiltshire *et al.*, 1995). While OA induces chromosome condensation in a variety of cell types (oocytes as well as mitotic cells), it was surprising that the chromosomes visualized after OA treatment of pachytene spermatocytes were chiasmate bivalents, since it had been assumed that the pachytene cells were in the process of recombination. Importantly, chromosomes that condensed after OA treatment were always bivalents and never univalents. Other characteristics of a normal G2/M transition were noted after OA treatment, including activation of the H1 kinase activity of MPF and disassembly of the SC. Because the condensed chromosomes visualized after OA treatment of pachytene spermatocytes exhibited normal numbers of chiasmata, it was concluded that the pachytene spermatocytes had already undergone events of recombination, or could quickly resolve them after OA treatment. In these experiments, OA served as a reagent to reveal the spermatocyte's meiotic competence, or ability to condense chiasmate bivalent chromosomes; the experiments demonstrated that MI competence arises before the actual G2/M transition occurs *in vivo*.

The goal of the current study was to determine when spermatocytes become competent to condense MI chromosomes. The experiments were designed to test two hypotheses: that meiotic competence is acquired when homologous chromosomes achieve complete synapsis (at the onset of the pachytene stage) and that competence is associated with appearance of M-phase regulators in spermatocytes. Cells that are competent to condense MI chromosomes were compared with those that are not. This was done by taking advantage of techniques to isolate two cell populations enriched for spermatocytes from 17-day-old mice. The first is a fraction of pachytene spermatocytes, exhibiting complete homologous chromosome synapsis, but which, at this age, have not advanced to the end of the pachytene stage. The second is a population of leptotene/zygotene (L/Z) spermatocytes (Bellvé *et al.*, 1977), consisting of spermatocytes that have not begun, or have begun but not completed, chromosome synapsis: the leptotene and zygotene spermatocytes, respectively. By comparing the ability of pachytene and L/Z spermatocytes to condense MI chromosomes after OA treatment, it was shown that the ability to condense MI chromosomes (MI competence) arises after the zygotene stage. The timing of the acquisition of MI competence was more precisely determined by isolating germ cells from prepuberal mice of progressively greater ages during the first wave of spermatogenesis. The developmental acquisition of MI competence occurs at midpachynema and is correlated with developmental accumulation of regulators of the cell cycle.

MATERIALS AND METHODS

Animals, Preparation of Cells, and Culture Procedures

ICR mice were obtained from Harlan (Indianapolis, IN) and maintained under standard conditions. For enrichment of leptotene/zygotene spermatocytes and juvenile pachytene spermatocytes, testes from approximately seventy 17-day-old mice were excised and the germ cells separated on a 2–4% bovine serum albumin gradient as described (Bellvé *et al.*, 1977). This procedure yielded L/Z spermatocyte and juvenile pachytene spermatocyte fractions of >75% purity as assessed by morphological criteria under Nomarski optics and verified by indirect immunofluorescence analysis of the synaptonemal complex of fixed aliquots of

cells (Dobson *et al.,* 1994). Culture conditions were as previously described for pachytene spermatocytes from adult mice (Handel *et al.,* 1995). Cells were cultured at 2.5×10^6 cells/ml at 32°C, 5% CO_2. After a 1-h acclimation period cells were treated with 5 μM OA (ICN) added from a stock of 244 μM dissolved in ethanol. Control cultures received an equivalent amount of ethanol alone. After a 6-h culture period cells were harvested and analyzed as described below.

Mixed germ cells were prepared from testes of mice of ages 7–17 days, using three to five mice of each age per experiment. Detunicated testes were digested in 0.5 mg/ml collagenase (Sigma) for 20 min at 32°C and then 0.5 mg/ml trypsin (Sigma) for 13 min as previously described (Bellvé, 1993). The germ cells were filtered through a 53-μm mesh and washed three times in Kreb's–Ringer bicarbonate solution before culture. This mixed population of germ cells was cultured as were the enriched fractions described above. An aliquot of the cells was fixed for immunocytological analysis of meiotic stages. Cells (2.5×10^6/ml) were treated with 5 μM OA or ethanol alone after a 1-h acclimation period. Cells were analyzed for meiotic progression as described below after a 6-h treatment period.

Cytological Methods

Cells were fixed for indirect immunofluorescence analysis in 2% paraformaldehyde, 0.03% SDS as previously described (Cobb *et al.,* 1997). Polyclonal rabbit anti-hamster SYN1, recognizing mouse SYCP1, polyclonal mouse anti-hamster COR1, recognizing mouse SYCP3, and polyclonal rabbit anti-histone H1t were kindly provided by Dr. Peter Moens, York University. Anti-phospho histone H3 was obtained from Upstate Biotechnology (Lake Placid, NY). Secondary antibodies were purchased from Pierce (Rockford, IL) and conjugated with either rhodamine or fluorescein. DAPI was added to the mounting medium (Prolong Antifade, Molecular Probes, Eugene, OR) at 0.1 μg/ml to stain DNA. Immunofluorescence was observed with a 100× objective of an Olympus epifluorescence microscope. Images were captured to Adobe Photoshop with a Hamamatsu C5810 color chilled CCD camera. Nuclei were scored for meiotic stage by previously described criteria (Dobson *et al.,* 1994).

To stage the proportion of cells which had progressed to MI, a modification of the Evans procedure was used (Evans *et al.,* 1964) as previously described (Wiltshire *et al.,* 1995). Air-dried, Giemsa-stained preparations of cells were scored for the presence of MI figures or other degrees of chromatin condensation by brightfield observation with a 40× objective.

MPF Assay

The assay of CDC2/CYCLIN B (MPF) in spermatocytes was a modification of previously described procedures (Rhee and Wolgemuth, 1997; Zhu *et al.,* 1997). At 0, 2, 4, and 6 h, one milliliter of cultured cells, 2.5×10^6 L/Z or pachytene spermatocytes, was collected by centrifugation, washed twice, and frozen at −80°C. Upon thawing, the cells were lysed in 100 μl of lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml DNase) for 30 min on ice. Chemicals were obtained from Sigma (St. Louis, MO). The cells were further lysed by homogenization with a microcentrifuge pestle and then centrifuged at 14,000*g* at 4°C for 10 min. The supernatant was taken as the protein lysate. Protein concentrations of lysates were determined by the Bradford method (Bio-Rad, Hercules, CA). MPF was precipitated by incubating 50 μg of the protein lysate with 25 μg $p13^{suc1}$ (p13) agarose beads (Upstate Biotechnology) for 2 h at 4°C. The p13 beads were collected by centrifugation at 14,000*g* at 4°C for 20 s. The beads were washed three times in 500 μl of the lysis buffer followed by a single wash in the assay buffer (25 mM MOPS, pH 7.2, 60 mM β-glycerol phosphate, 30 mM *p*-nitrophenylphosphate, 15 mM EGTA, 15 mM $MgCl_2$, 100 μM Na_3VO_4, 1 mM DTT, 1 mM PMSF). The beads were resuspended finally in 20 μl kinase buffer and duplicate 5-μl samples were used immediately in the histone H1 kinase assay. The kinase assay included 5 μl of the p13 precipitate or 5 μl lysate in a total volume of 25 μl assay buffer including 100 μg/ml histone H1 (type III-S, Sigma), 15 μM ATP, 1 μg/ml cAMP-dependent protein kinase inhibitor peptide, and 100 μCi/ml [γ-^{32}P]ATP. Reactions were incubated for 1 h at 37°C and stopped by addition of 25 μl 2× Laemmli sample buffer. Assay mixes were resolved on 12.5% SDS–PAGE followed by autoradiography. Phosphorylated histone H1 was quantitated by scanning densitometry of autoradiograms and/or scintillation counting of the excised bands following electrophoresis.

Immunoblot Analysis

Cells were lysed as described for the MPF assay. Ten micrograms of total protein or 10 μl of p13 precipitates was separated on 10% SDS–PAGE and transferred to nitrocellulose using a Bio-Rad semi-dry transfer apparatus. After transfer for 30 min the blots were blocked for 1 h at room temperature or overnight at 4°C in PBS, 0.1% Tween 20, 3% BSA. The blocked blots were probed with monoclonal anti-CDC2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) , monoclonal anti-CYCLIN B1 (kindly provided by Julian Gannon, ICRF), polyclonal anti-HSP70-2 serum 2A (kindly donated by E. M. Eddy, NIEHS), MPM-2 monoclonal (Upstate Biotechnology), or a rabbit polyclonal anti-hamster CDC25C, provided by K. Yamashita (Seki *et al.,* 1992), each diluted in blocking buffer. After washing with PBS–0.1% Tween 20, the blots were probed for 1 h with horseradish peroxidase-conjugated secondary antibody (Pierce) diluted at 1:1000 to 1:20,000 in blocking solution. After washing, the blots were incubated with ECL chemiluminescent substrate (Amersham, Arlington Heights, IL) and exposed to film according to the manufacturer's instructions.

Immunohistochemistry

Testes from adult ICR mice were excised and fixed in Bouin's solution (Sigma) for 6 h. Fixed testes were dehydrated and embedded in paraffin according to standard procedures. Embedded testes were sectioned at 5 μm and placed on albuminized slides. After allowing the slides to dry, the tissue was deparaffinized and rehydrated, immediately followed by boiling in 10 mM sodium citrate buffer (pH 6.0) for 15 min as described (Mizoguchi and Kim, 1997). Slides were washed for 10 min in PBS and blocked for 20 min in 10% goat serum, 10 μg/ml DNase in PBS. Slides were then rinsed with 10% goat serum to remove excess DNase. Slides were incubated overnight at 4°C with 1:150 polyclonal anti-CDC25C (Santa Cruz), or the polyclonal anti-hamster CDC25C as used in Western blotting, in 10% goat serum/PBS. The following day slides were rinsed 3× 10 min in PBS and incubated with 1:100 rhodamine-conjugated secondary antibody (Pierce) for 2 h at room temperature. Controls included reaction with normal rabbit serum and, when possible, reaction

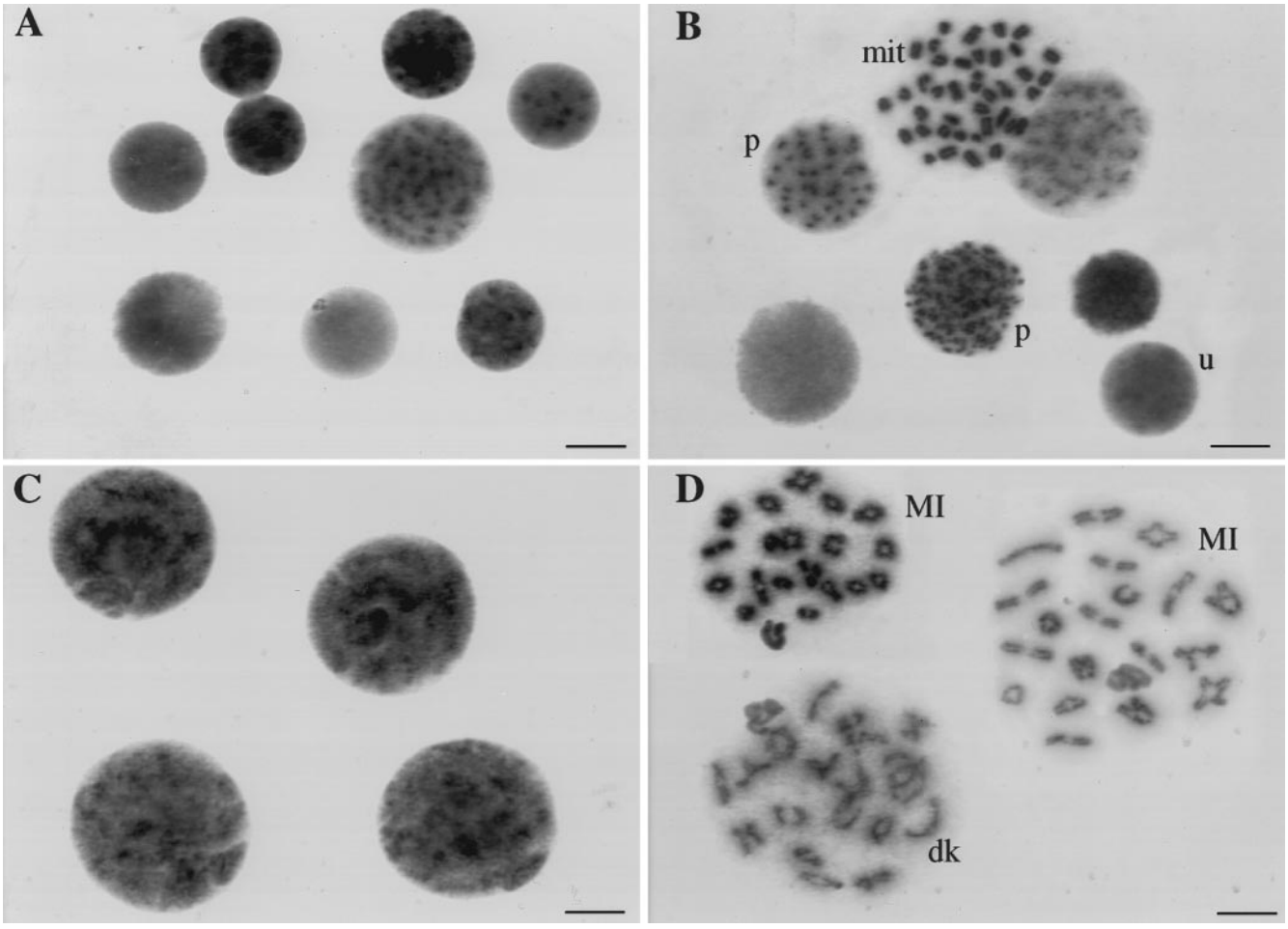

FIG. 1. Air-dried nuclear preparations showing chromatin configuration of spermatocytes from 17-day-old mice after 6 h of culture in the presence or absence of OA. (A) L/Z cells cultured without OA. Chromatin in all nuclei appears uncondensed. (B) L/Z spermatocytes cultured with 5 μM OA. Chromatin was either scored as uncondensed (u), punctate (p), or mitotic (mit). (C) Pachytene spermatocytes cultured without OA. Chromatin is uncondensed except for visible sex bodies. (D) Pachytene spermatocytes cultured for 6 h with 5 μM OA. Most cells have condensed chromatin to a metaphase I (MI) or diakinesis (dk) configuration with visible chiasmata. Bars = 10 μm.

with primary antibody absorbed with immunizing peptide. Slides were then washed 3× 10 min with PBS and mounted with Prolong Antifade as described above.

RESULTS

Leptotene/Zygotene Spermatocytes Are Not Competent to Condense MI Chromosomes

Earlier studies (Wiltshire *et al.*, 1995) showed that cultured pachytene spermatocytes from adult mice are competent to condense chiasmate bivalent chromosomes when treated with OA. To define when spermatocytes become competent to condense MI chromosomes, fractions of L/Z spermatocytes and juvenile pachytene spermatocytes were enriched from 17-day-old mice. When treated with 5 μM OA approximately 75% of the juvenile pachytene spermatocytes condensed MI chromosomes just as did pachytene spermatocytes from adult mice (Fig. 1 and Table 1). However, the L/Z spermatocytes did not condense MI chromosomes in response to 5 μM OA treatment. Although a significant number (approximately 42%) of the L/Z cells did show some chromatin condensation, the chromatin did not form an MI configuration and chromosomes or chromosome-like structures were not visible (Fig. 1B). Approximately 10% of cells from the L/Z fraction condensed univalent, mitotic-like chromosomes; this pattern of condensation could result from contaminating spermatogonia or some subfraction of the L/Z spermatocytes. This result defines a difference between L/Z and pachytene spermatocytes in MI competence.

TABLE 1
Effects of OA on Chromatin Configuration of Cultured Spermatocytes from 17-Day-Old Mice

Cell fraction	Treatment (6 h)	Chromatin configuration			
		Uncondensed	MI/diakinesis	Mitotic	Punctate
L/Z	Control	97.4 ± 1.1[a]	0	2.4 ± 0.7	1.0 ± 0.6
L/Z	5 μM OA	56.6 ± 6.4	0.1 ± 0.06	10.1 ± 2.7	32.3 ± 3.7
Pachytene	Control	97.9 ± 0.7	1.1 ± 0.5	0.6 ± 0.2	0
Pachytene	5 μM OA	16.7 ± 1.0	75.7 ± 3.7	2.3 ± 0.6	0

Note. L/Z, leptotene/zygotene spermatocyte fraction.
[a] These data indicate the mean percentages of cells (±SEM) in each configuration from four separate experiments. At least 500 cells were scored per experiment.

MI Competence Appears during Mid-pachynema

Does competence coincide with the establishment of full chromosome synapsis at the beginning of pachynema? To define more precisely the timing of competence, we took advantage of the synchronous first wave of spermatogenesis in prepuberal mice. By isolating germ cells of mice of progressively greater age, germ cells of progressively later stages are obtained, thus permitting a temporal order of meiotic events to be established. Enzymatic digestion was used to obtain populations of mixed germ cells at daily intervals from mice between 7 and 17 days of age. Germ cells were cultured, treated with OA, and scored for the appearance of MI chromosomes to determine at which stage spermatocytes acquire MI competence, as shown in Fig. 2 and Table 2.

Indirect immunofluorescence staining of germ cell nuclei with COR1, SYN1, and H1t antibodies was used to stage cell types, and thus correlate stage to response to OA. Pachytene spermatocytes were clearly present by day 11 and made up close to 10% of germ cells by day 14 (Fig. 2 and Table 2). However, significant numbers of cells condensing MI chromosomes in response to OA were not found until day 15.

Interestingly, acquisition of MI competence temporally coincides with the appearance of the testis-specific histone H1t in pachytene spermatocytes (Fig. 2). Previous studies had shown that histone H1t first appears at mid-pachynema (Drabent *et al.*, 1996).

MPF in Leptotene/Zygotene Spermatocytes Is Not Activated by Okadaic Acid

In all cells that have been studied, cell-cycle progression to metaphase is regulated by activation of MPF, a complex of CDC2 kinase and its regulatory subunit CYCLIN B1. We, and others, have shown that OA treatment activates MPF activity in pachytene spermatocytes from adult mice (Rhee and Wolgemuth, 1997; Wiltshire *et al.*, 1995). Here, we assayed H1 kinase activity in the p13 precipitate from cell lysates. p13 precipitates are routinely used to assay MPF (Rhee and Wolgemuth, 1997); however, these precipitates may also include other cyclin-dependent kinases (Meyerson *et al.*, 1992). The H1 kinase activated by OA has been shown to be CDC2 in mitotic cells (Yamashita *et al.*, 1990). Nonetheless, it should be noted that some fraction of this activity could be due to CDK2 or other cyclin-dependent kinases, although CDK2 has not been shown to be activated by OA. Figure 3 shows greater than fivefold activation of H1 kinase in p13 precipitates in OA-treated pachytene spermatocytes, but no activation in OA-treated L/Z spermatocytes. In addition to the activity measured after 6 h of OA treatment (Fig. 3), no histone H1 kinase activation in p13 precipitates of L/Z spermatocytes was observed after 2 or 4 h of OA treatment (data not shown), suggesting complete failure of OA to activate MPF. These data implicate a biochemical difference between L/Z and pachytene spermatocytes in the pathway leading to MPF activation.

Leptotene/Zygotene Spermatocytes Contain CYCLIN B1 and CDC2, but Not CDC25C

Because L/Z and pachytene spermatocytes differed in their ability to activate MPF, Western blotting was employed to detect the components of MPF and its regulators in these cell fractions (Figs. 4A and 4B). We used p13 agarose beads to precipitate proteins associated with CDC2 (Dunphy *et al.*, 1988). CDC2 and its regulatory subunit CYCLIN B1 were detected in lysates from both L/Z and pachytene spermatocytes, and both proteins were precipitated with p13 agarose, suggesting that the MPF complex is already present in both cell types. This is consistent with the presence of basal (not OA-activated) MPF activity in both cell types (Fig. 3).

The testis-specific molecular chaperone HSP70-2 has previously been shown to be required for the assembly of the MPF complex in spermatocytes (Zhu *et al.*, 1997). HSP70-2 was detected in both L/Z and pachytene spermatocytes, consistent with the presence of MPF in both cell types (Fig. 4C). However, p13 precipitated HSP70-2 only in pachytene spermatocytes and not in L/Z spermatocytes (Fig. 4C). Zhu *et al.* (1997) reported that HSP70-2 associates

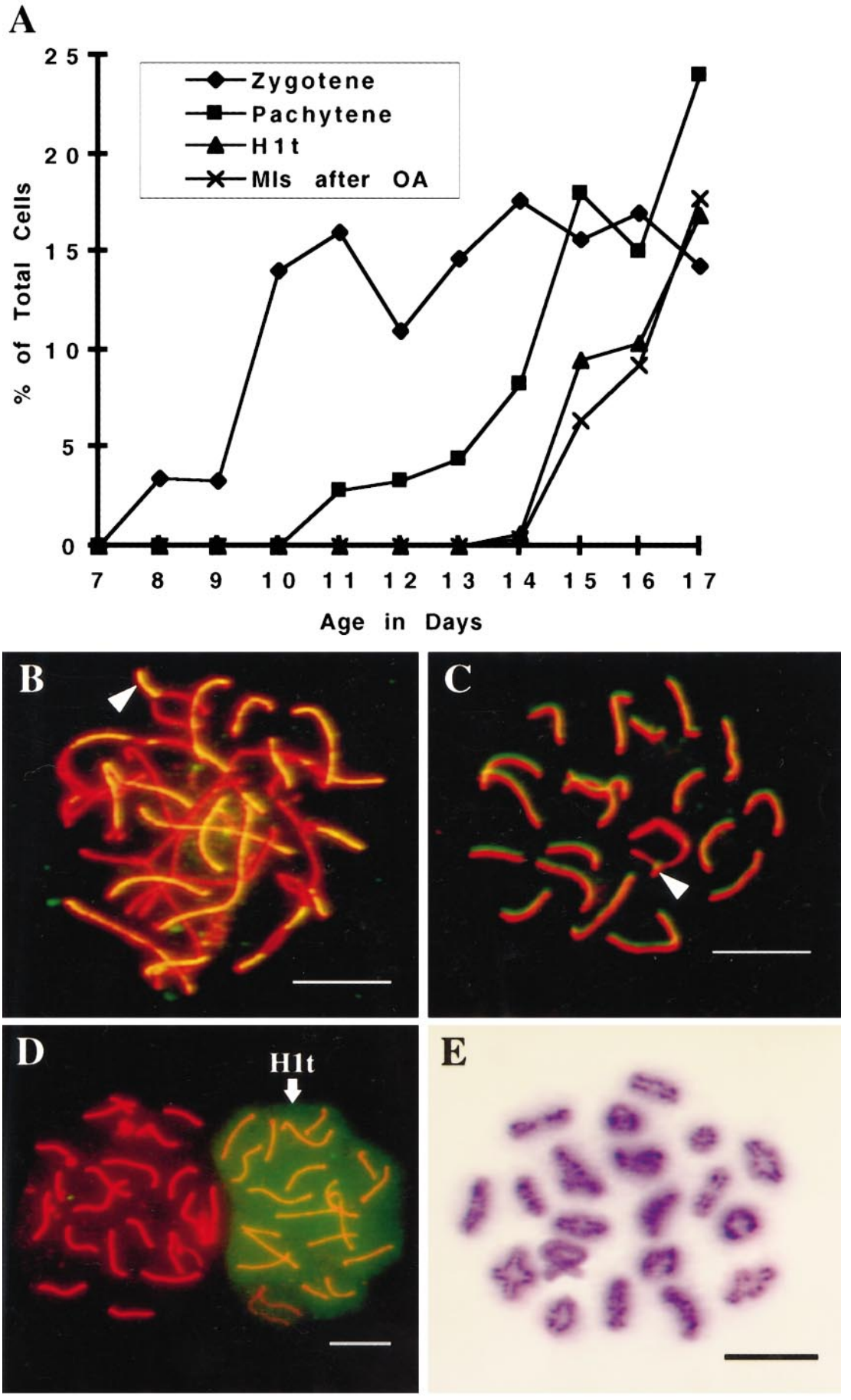
A
25
20
15
10
5
0
% of Total Cells
Zygotene
Pachytene
H1t
MIs after OA
7
8
9
10
11
12
13
14
15
16
17
Age in Days
B
C
D
H1t
E

TABLE 2
Scoring of Cell Types and Presence of H1t and MI Competence in Germ Cells of Prepuberal Mice

Age of mice (days)	Category			
	Zygotene	Pachytene	H1t-postive cells	MIs after OA
13	13.5 ± 1.1[a]	3.8 ± 0.6	0	0
14	14.2 ± 3.4	8.3 ± 0	0.5 ± 0.1	0.2 ± 0.2
15	13.6 ± 1.5	18.0 ± 0.1	7.2 ± 2.3	4.4 ± 2.0
16	16.4 ± 0.6	18.1 ± 3.0	12.6 ± 2.3	7.0 ± 2.2

[a] These data represent the mean percentages of cells (±SEM) in each category from all germ cells isolated in two separate experiments.

with CDC2 only before assembly of MPF. Therefore, presence of HSP70-2 in the p13 precipitates from pachytene spermatocytes implies that some CDC2 may not yet be complexed with cyclin in these cells.

Dephosphorylation of CDC2 by CDC25C protein phosphatase leads to its activation (Gautier *et al.*, 1991). Experiments were conducted to detect this activating phosphatase in both incompetent L/Z and competent pachytene spermatocytes. A polyclonal antiserum detected a band at the appropriate molecular weight in lysates from pachytene spermatocytes, but not in L/Z spermatocytes (Fig. 4D).

Progression to metaphase in mitotic cells is accompanied by the appearance of specific phosphoepitopes, designated the MPM-2 antigens (Davis *et al.*, 1983). Appearance of these phosphorylated antigens has been shown to occur with OA treatment in other cell types (Yamashita *et al.*, 1990). Of particular relevance, acquisition of meiotic competence by mouse oocytes has been shown to correlate with appearance of MPM-2 antigens (Wickramasinghe *et al.*, 1991). A dramatic increase in MPM-2 antigen was found in both incompetent L/Z spermatocytes and competent pachytene spermatocytes treated with OA (Fig. 4E). Therefore accumulation of MPM-2 phosphoepitopes is not dependent on MPF activation (which does not occur in L/Z spermatocytes) nor is it sufficient to cause progression to MI (which also does not occur in L/Z spermatocytes). Nonetheless, appearance of the MPM-2 antigen is not simply a nonspecific effect of OA treatment since similarly treated round spermatids did not accumulate significant MPM-2 antigens (Fig. 4E).

CDC25C Appears during Mid-pachynema

L/Z spermatocytes are deficient in the MPF activator CDC25C (Fig. 4D). Since it had been determined that MI competence is acquired in mid-pachynema (Fig. 2), experiments were conducted to define the appearance of CDC25C with greater precision, to determine how tightly coupled it was with the acquisition of competence. Others have used *in situ* hybridization to show that the mRNA for CDC25C is present in late pachytene spermatocytes (stage IX) and round spermatids in mice (Wu and Wolgemuth, 1995). Mizoguchi and Kim (1997) found that immunostaining for CDC25C increased through pachynema in the rat, but the precise stage of its appearance was not reported. The time of appearance of CDC25C protein during spermatogenesis was determined by staining testis sections from adult mice with an antibody specific for CDC25C (Fig. 5). CDC25C staining was not seen in pachytene spermatocytes from stage I through stage IV of the seminiferous epithelium, although

FIG. 2. Sequential appearance of cell types, histone H1t, and MI competence in prepuberal mice. Germ cells were isolated from three to five mice of identical age on each day between 7 and 17 days of age. (A) Zygotene, pachytene, and cells positive for H1t were scored by indirect immunofluorescence in samples from each age. The total number of DAPI-positive cells was counted using a 100× objective, and the number in zygonema or pachynema and expressing histone H1t was determined. At least 200 cells were scored for each sample. Air-dried, Giemsa-stained nuclear preparations of cells which had been cultured with 5 μM OA were scored for the presence of MI nuclei using brightfield observation. In this case at least 500 cells were scored per sample. For clarity, only one set of data is shown, but the experiment was repeated for the critical 13–16 days of age, and the means with SEM of both experiments are shown in Table 2. (B–D) Examples of cells, bars = 10 μm. (B) This image shows a zygotene spermatocyte from a 9-day-old animal stained with COR1 and SYN1. Fully synapsed regions are yellow due to overlap of green and red signals (arrowhead). The red COR1 signal is on unsynapsed regions, indicating that this cell is a zygotene spermatocyte. (C) This image shows a pachytene spermatocyte from a 12-day-old animal. The SYN1 (green) and COR1 (red) images were slightly offset to show more clearly that they completely overlap, indicating full synapsis and therefore a pachytene cell. The sex chromosomes synapse only in the pseudoautosomal region (arrowhead). (D) These are two pachytene spermatocytes from a 16-day-old animal stained with COR1 (red) and anti-H1t (green). The cell on the left is negative for H1t and is therefore an early pachytene spermatocyte. H1t-positive pachytene cells appeared at age 15 days. (E) This is an MI nucleus induced by OA treatment of cultured germ cells from a 15-day-old animal.

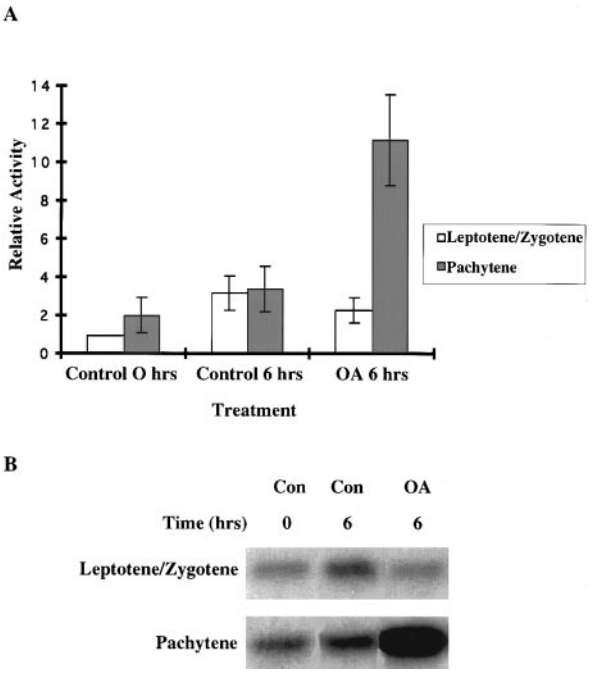

FIG. 3. Effect of OA treatment on H1 kinase activity in p13 precipitates from cultured L/Z and pachytene spermatocytes from 17-day-old animals. Spermatocytes were cultured for 6 h in the presence of 5 μM OA, or solvent alone (control). MPF was precipitated from cell lysates with p13 agarose beads and the activity determined by the ability of precipitates to phosphorylate histone H1. (A) This graph represents the mean H1 kinase activity in five repeat experiments with SEM shown. Phosphorylation was quantitated by scanning densitometry of autoradiograms, and the relative activity was determined by dividing the density of the band by that of the control L/Z band at $T = 0$. Scintillation counting of excised bands gave similar results. (B) Autoradiogram showing phosphorylated histone H1 from one representative experiment. Con, control culture treated with solvent alone; OA, 5 μM okadaic acid-treated culture.

the protein is present in round spermatids at this stage. A low level of CDC25C staining was first detected in mid-pachytene spermatocytes at stage V of the seminiferous epithelium (not shown); staining increased in later stages and was intense at stage X (Fig. 5B). Stage X L/Z spermatocytes were negative for CDC25C staining, in agreement with results from Western blots. A similar staining pattern was seen with the same polyclonal antibody used in the Western blotting experiment in Fig. 4C (not shown). The CDC25C protein was detected much earlier than the corresponding RNA was detected in a previous study (Wu and Wolgemuth, 1995). This discrepancy may be due to differences in the sensitivity of the two methods or the relative abundance of the RNA and protein. Therefore, CDC25C is first detected at the same time as acquisition of MI competence, during mid-pachynema.

Histone H3 Phosphorylation Is Not Sufficient for Condensation of MI Chromosomes

Although CDC2 is not activated by OA treatment in L/Z spermatocytes (Fig. 3), Western blotting with the MPM-2 antibody showed that mitotic-specific phosphorylation events occur nonetheless (Fig. 4E). Phosphorylation of histone H3 on serine 10 has been strongly correlated with the condensation of mitotic chromosomes, and this phosphorylation has been postulated to be an essential element contributing to mitotic chromosomal condensation (Hendzel *et al.*, 1997). Indirect immunofluorescence was used to examine the phosphorylation status of histone H3 in both incompetent and competent spermatocytes, with and without OA treatment (Fig. 6). Neither pachytene nor L/Z spermatocytes contained phosphorylated histone H3 in control cultures (Figs. 6A and 6C). In untreated spermatocytes, H3 phosphorylation was first observed at the diplotene stage (not shown). However, after OA treatment, both L/Z and pachytene spermatocytes showed a dramatic appearance of histone H3 phosphorylation (Figs. 6B and 6D). Thus, although L/Z spermatocytes can phosphorylate histone H3, this phosphorylation event is not sufficient to induce the condensation of MI chromosomes in these cells. When CDC2 activity was blocked in pachytene spermatocytes by the cyclin-dependent kinase inhibitor butyrolactone I, histone H3 phosphorylation after OA treatment was not inhibited even though MI chromosome condensation was inhibited (data not shown). Taken together, these results imply that phosphorylation of histone H3 is not sufficient for condensation of MI chromosomes in spermatocytes.

DISCUSSION

Meiotic competence of mammalian oocytes has been assessed by their ability to progress spontaneously through meiosis after their removal from inhibitory follicular factors (Eppig, 1993; Handel and Eppig, 1998). Unlike competent oocytes, spermatocytes do not spontaneously enter the meiotic division phase when removed from the seminiferous tubule environment. Also unlike oocytes, spermatocytes progress through meiosis with no arrest stage. For these reasons, it has previously been difficult to assess experimentally when spermatocytes acquire competence to condense chiasmate MI bivalent chromosomes ("MI competence"). OA, which precociously induces meiotic chromosome condensation, was used as a tool to determine the timing of acquisition of MI competence by spermatocytes. It was found that spermatocytes acquire the ability to condense chiasmate bivalent chromosomes midway through the pachytene stage, coincident with the appearance of both the testis-specific histone H1t and the CDC25C protein phosphatase.

What does the acquisition of MI competence signify? Development of MI competence in the spermatocyte may depend on completion of genetic or biochemical events, or,

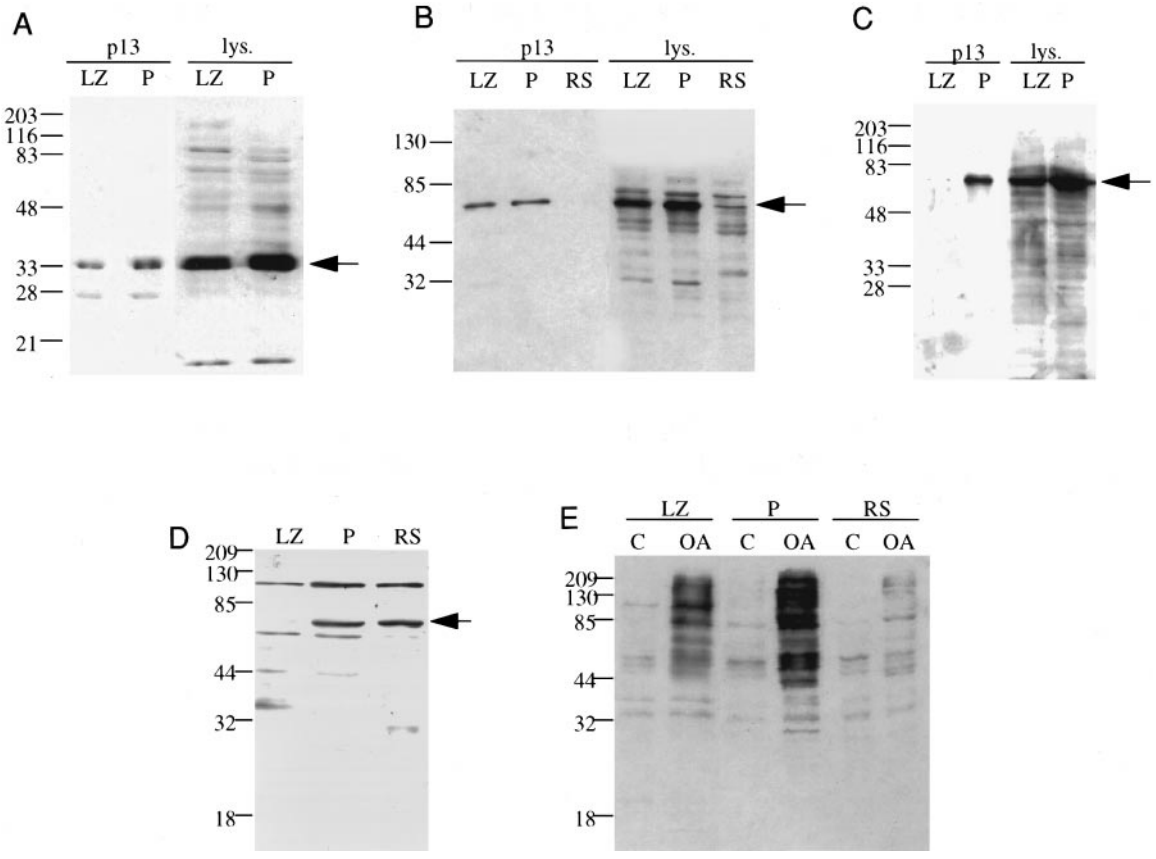

FIG. 4. Immunoblots of G2/M cell-cycle proteins in MI-competent (pachytene) and -incompetent L/Z cell fractions from 17-day-old mice. (A) CDC2 protein (arrow) in p13 precipitates and total cell lysates (lys.) from L/Z and pachytene (P) spermatocytes. CDC2 is found in cell lysates and p13 precipitates from both cell fractions. (B) CYCLIN B1 protein (arrow) is found in lysates and p13 precipitates from L/Z and pachytene cells, indicating that it is complexed with CDC2, but much reduced in the postmeiotic round spermatids (RS). (C) The testis-specific molecular chaperone HSP70-2 is detected in lysates from L/Z and pachytene cells, but in the p13 precipitates only from pachytene cells. (D) Antisera directed against CDC25C detect a band (arrow) in lysates from pachytene spermatocytes and round spermatids, but not L/Z cells. (E) Blot of lysates from 6 h control (C) or 5 μM OA-treated (OA) cultures of L/Z and pachytene spermatocytes and round spermatid cells probed with the MPM-2 antibody which recognizes a mitosis-specific phosphoepitope.

more probably, both. By our definition, MI competence means that genetic recombination has progressed sufficiently to allow homologous chromosomes to form condensed metaphase bivalents held together by chiasmata; this defines a level of "genetic competence." The hypothesis underlying this work was that MI competence is acquired when homologous chromosomes synapse completely; however, it was found that competence is not acquired until several days after synapsis, implying a requirement for other events. Additionally, the finding that incompetent L/Z spermatocytes differ biochemically from competent pachytene spermatocytes suggests that incompetent spermatocytes lack the necessary biochemical machinery for chromosome condensation, thus defining "biochemical competence." Genetic and biochemical competence are probably related; possibly the spermatocyte

FIG. 5. Indirect immunofluorescence labeling of CDC25C protein in tissue sections from adult mouse testes. (A) In this stage IV section, early pachytene spermatocytes (p) show no CDC25C staining (red), while round spermatids (rs) stain intensely. (B) In contrast, pachytene spermatocytes in a stage X section are positive for CDC25C, while neighboring leptotene spermatocytes (l/z) are negative. (C) To show the specificity of staining, this stage XI seminiferous tubule section was stained with CDC25C antiserum which was preadsorbed with the CDC25C immunizing peptide, which eliminated staining. D, E, and F show the DAPI staining patterns which were used to identify the cell types in A, B, and C, respectively. Bars = 10 μm.

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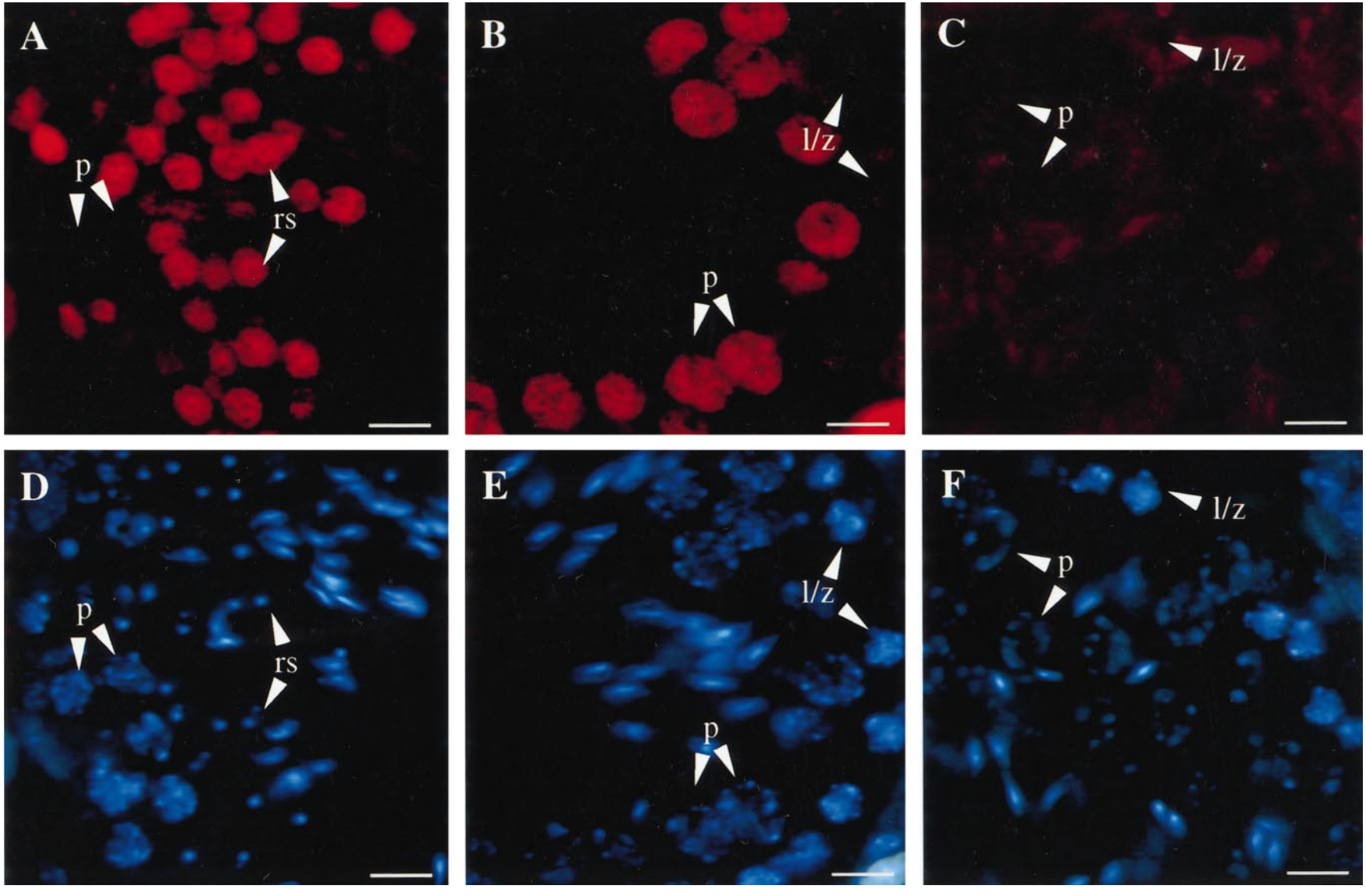

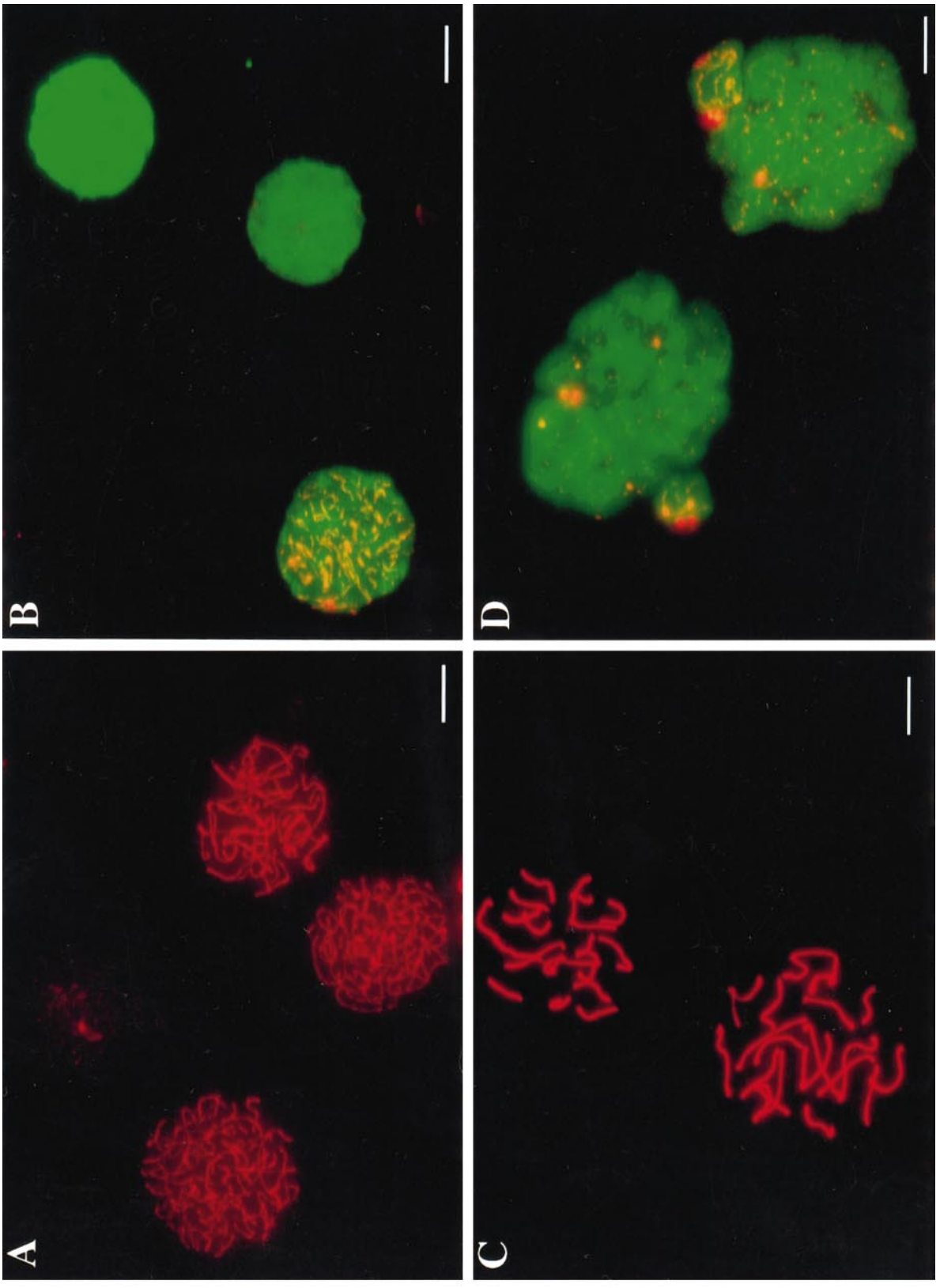

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monitors recombination events for completion. Coupling synthesis and assembly of cell-cycle regulators to the completion of recombination would ensure that the cell-cycle transition does not occur before assembly of chiasmata, which are required for accurate segregation of homologous chromosomes.

SYN1 and COR1 antibodies were used to time precisely the appearance of pachytene spermatocytes during development (Fig. 2). By using OA as a tool for assessing the time spermatocytes acquire MI competence, it was found that competence is acquired several days before the time spermatocytes enter the meiotic division phase *in vivo*. Thus, OA somehow bypasses another process that regulates entry into division phase *in vivo*. At this point there is no evidence indicating what that process may be, but it could be related to the dramatic growth phase that occurs in spermatocytes between the acquisition of MI competence at stage V and the actual meiotic division process at stage XII. These results permit some inferences about timing of events contributing to the genetic and biochemical aspects of competence.

Genetic Competence: Chiasmata Formation during Meiosis

Competence to condense chromosome bivalents held together by chiasmata, which are the visible manifestation of prior recombination events, is acquired midway through pachynema. This observation is one of the first bits of evidence about the timing of recombination events during prophase in mammalian meiosis. Although little is known about the nature of the events that lead to chiasmata formation in mammals, much is known about the molecular biology of meiosis in the yeast *Saccharomyces cerevisiae* (Kleckner, 1996; Roeder, 1997). In budding yeast, recombination is initiated by the generation of double-strand breaks during leptonema, catalyzed by the product of the *SPO11* gene (Keeney *et al.*, 1997). This occurs coincident with first assembly of lateral axes of the SC (Padmore *et al.*, 1991), leading to the idea that initiation of synapsis in zygonema is dependent on initiation of recombination in these cells. The recombination intermediates, double Holliday junctions, are detected early in pachynema, when, by definition, chromosomes are fully synapsed, but mature recombinant molecules are not detected until the end of pachynema (Schwacha and Kleckner, 1995). There is no clear evidence that the same events, or their sequence and timing, occur during mammalian meiosis, although conservation of processes is implied by finding mammalian homologs of genes important in yeast meiosis, including *Rad51* (Morita *et al.*, 1993) and *Dmc1* (Pittman *et al.*, 1998; Yoshida *et al.*, 1998). Our observations suggest that in mouse spermatocytes much of recombination is completed, or at the point where it can be rapidly resolved to form chiasmata, by mid-pachynema.

While it is tempting to view the recombined chromosomes and chiasmata seen after OA treatment as "normal" because they are cytologically similar to chromosomes seen in MI spermatocytes, it is known only that the chiasmata are functional in holding the members of the bivalent together. It is not known if these chiasmata, visualized in mid-pachytene spermatocytes by virtue of OA treatment, represent mature recombination events, with mismatch and repair synthesis completed. Nor is it known whether these chiasmata can function to provide correct metaphase orientation on the spindle or to ensure accurate reductional segregation of homologs at the first meiotic anaphase. In fact, evidence suggests that the chiasmata that are formed by pachytene spermatocytes stimulated precociously to meiotic division by injection into oocyte cytoplasm may not function correctly in ensuring orientation and segregation. Such injected primary spermatocyte nuclei undergoing meiotic division in the oocyte exhibit many abnormalities of chromosome behavior, including chromosome lagging, fragmentation, and premature sister chromatid separation (Kimura *et al.*, 1998; Ogura *et al.*, 1998).

Biochemical Competence: Proteins Mediating Cell Cycle and Chromatin Condensation

The presence of the universal regulators of the cell cycle has been detected previously in spermatogenic cells at both the transcript and protein level (Wolgemuth *et al.*, 1995). However, the biological significance of the timing of their appearance and their relationship to progress of meiotic events have remained elusive. This study provides correlation between the appearance and activation of cell-cycle proteins and acquisition of competence to condense chiasmate bivalents in meiosis. It had previously been shown that there is MPF activity in pachytene spermatocytes (Chapman and Wolgemuth, 1994; Wiltshire *et al.*, 1995), and Western blotting was used to detect the components of MPF (CDC2 and CYCLIN B1) in pachytene spermatocytes (Chapman and Wolgemuth, 1994). Our results extend these

FIG. 6. Effect of OA treatment on histone H3 phosphorylation in L/Z and pachytene spermatocytes cultured for 6 h. Cells from 17-day-old animals were enriched, treated, and cultured as in Fig. 1. Nuclei in each image were labeled with anti-COR1 (red) and anti-phosphorylated histone H3 (green). For comparison purposes, all images had identical exposure times with the FITC filter (green). (A) Control cultured L/Z spermatocytes, showing forming axial elements of the SC, but no histone H3 phosphorylation. (B) OA-treated L/Z spermatocytes showing histone H3 phosphorylation throughout their nuclei. (C) Control cultured pachytene spermatocytes, with anti-COR1 staining showing fully formed SCs, but no histone H3 phosphorylation. (D) OA-treated pachytene spermatocytes, which have progressed to an MI configuration, showing histone H3 phosphorylation throughout their chromatin. Bars = 10 μm.

findings and show that MPF components are present at the earlier L/Z stages as well, but cannot be activated by OA until the pachytene stage.

The difference found here between L/Z and pachytene spermatocytes in OA-mediated activation of MPF led to analysis of the CDC2-activating phosphatase CDC25C. Wu and Wolgemuth (1995) had previously shown that CDC25C is present in pachytene spermatocytes from adult mice and is also present in whole testis extracts from 19-day-old mice. Here we have defined more precisely both the cells that have CDC25C and the developmental timing of its appearance by immunostaining sections of adult testes. CDC25C is not present in prophase cells which are incompetent (L/Z and early pachytene spermatocytes); this observation supports the hypothesis that this protein is required for MI competence. Those cells in which CDC25C is first expressed are those that first become competent to enter MI given the stimulus by OA. However, it is important to understand that these competent cells do not enter MI spontaneously in the testis; they spend several more days in meiotic prophase before passing through diplotene to MI. Thus, the critical regulation of the G2/M transition must be at some level other than expression of CDC25C protein, such as localization or activation (and even may not involve CDC25C). CDC25C has been shown to be activated by hyperphosphorylation of its C-terminal domain (Gabrielli *et al.*, 1997; Kumagi and Dunphy, 1992). The inactivating phosphatase that dephosphorylates CDC25C has been identified as PP2A in *Xenopus* oocyte extracts (Clarke *et al.*, 1993). These observations led to a model proposing that OA leads to MPF activation by inhibiting PP2A, resulting in maintenance of a hyperphosphorylated, constitutively active state of the CDC25C protein phosphatase, leading to MPF activation. This model may be valid for spermatocyte chromosome condensation in response to OA, although activation of CDC25C by phosphorylation has yet to be demonstrated in spermatocytes. *In vivo,* activation of CDC25C might be involved in signaling from recombination complexes to cell-cycle effectors in prophase spermatocytes. Recent studies link the ATM-dependent DNA damage checkpoint to mitotic progression through CDC25C in both mammalian and yeast cells (Furnari *et al.*, 1997; Sanchez *et al.*, 1997), with DNA damage leading to inhibitory phosphorylation of CDC25C by the CHK1 kinase. A role for ATM in spermatogenesis has been demonstrated by meiotic arrest of spermatocytes in the *Atm*−/− knockout (Xu *et al.*, 1996), and thus a similar checkpoint mechanism may act during spermatogenesis to monitor recombination-related DNA strand breaks and couple their repair to onset of metaphase. This hypothesis is supported by the observation that CHK1 associates with the synaptonemal complex in spermatocytes in an ATM-dependent manner (Flaggs *et al.*, 1997). To define conclusively the role of CDC25C in the spermatocyte meiotic cell-cycle transition, methods must be devised to assess more directly the role of CDC25C in spermatocytes.

Our observations correlate with those made in other species, especially *Drosophila,* where the spermatogenic meiotic role of the CDC25 homolog *twine* has been established using genetic tools (White-Cooper *et al.*, 1993; Maines and Wasserman, 1998). Although genetic competence, as we have defined it, by resolution of recombination, is not relevant for male *D. melanogaster,* where there is no chromosome synapsis and recombination, there are similar biochemical requirements for condensation and segregation of the asynaptic chromosomes. Spermatocytes in *twine* mutants partially condense chromosomes, but the chromosomes do not assemble at the metaphase plate, and the meiotic divisions do not occur. Therefore, *twine* apparently is required for normal meiotic metaphase in *Drosophila.* A temperature-sensitive mutant of the *Drosophila* homolog of CDC2, *Dmcdc2,* has an identical effect on spermatogenesis as does *twine,* indicating that *Dmcdc2* is likewise required for the meiotic divisions (Sigrist *et al.*, 1995). Interestingly, even though spermatocytes in the *twine* and *Dmcdc2* mutants do not divide, they do proceed through the process of spermiogenesis, producing inviable tetraploid spermatids, thus illustrating an uncoupling of the normally sequential processes of meiosis and spermiogenesis.

While the role of MPF in governing the G2/M transition is well established in a variety of cells, its requirement for chromosome condensation is controversial. The observation that spermatocytes of *Drosophila twine* and *Dmcdc2* mutants partially condense chromatin indicates that chromosome condensation is not wholly dependent on these gene products. Similarly, in the murine FT210 cell line, OA can induce the condensation of mitotic chromosomes in the absence of MPF activity, since treatment with OA or another phosphatase inhibitor, fostriecin, led to chromatin condensation in the absence of MPF activity (Guo *et al.*, 1995). Interestingly, some chromatin condensation was observed in some L/Z spermatocytes after OA treatment (Fig. 1) in the absence of activation of MPF (Fig. 3). If L/Z spermatocytes cannot activate MPF in response to OA treatment, what is responsible for the partial chromosome condensation observed? Other kinases, such as the mitogen-activated protein kinases (MAPK), may play a role. Evidence exists for a possible role for MAPK in oocyte germinal-vesicle breakdown (Chesnel and Eppig, 1995), and MAPKs are present in incompetent L/Z spermatocytes (unpublished observations). Phosphorylation of histone H3, which was independent of MPF activity, is correlated with the mitotic condensation of chromosomes in FT210 cells (Guo *et al.*, 1995), leading to the suggestion that chromosome condensation might be caused by histone H3 phosphorylation. This possibility was investigated, with the finding that histone H3 is phosphorylated after OA treatment in both competent pachytene and incompetent L/Z spermatocytes (Fig. 6). Significantly, however, even though histone H3 was phosphorylated, L/Z spermatocytes did not condense individualized chromosomes in response to OA. Thus, these results suggest a requirement for MPF activity in the condensation of meiotic chromosomes in spermato-

cytes, a hypothesis supported by our observations that inhibitors of MPF also inhibit chromosome condensation by pachytene spermatocytes in response to OA (unpublished observations). Clearly, multiple phosphorylation events occur in both L/Z and pachytene spermatocytes after OA treatment, as revealed by staining with the MPM-2 antibody (Fig. 4E), and this phosphorylation could lead to different degrees of chromosome condensation even in the absence of MPF activation. However, neither histone H3 phosphorylation nor the presence of MPM-2 phosphoepitopes is sufficient for assembly of individualized MI chromosomes.

In this study, staining with antibody against the testis-specific histone H1t was used to stage cells and discriminate early versus mid-to-late pachytene spermatocytes. Interestingly, there is a strong temporal correlation between the acquisition of MI competence and the appearance of H1t in spermatocytes; it is not yet known if there is a functional correlation. Somatic cell histone H1 is an *in vitro* substrate for MPF and there is good evidence that it is an *in vivo* substrate as well (Nigg, 1993) suggesting the possibility that H1t might govern MI competence. However, phosphorylation of H1t could not be detected in testis tissue (Khadake *et al.*, 1994). H1t associates more loosely with chromatin than does somatic histone H1 (Khadake *et al.*, 1994); it is possible that this association plays a role in remodeling chromatin to form MI chromosomes. H1t linker histone is expressed only in pachytene spermatocytes and spermatids and does not appear in pachytene spermatocytes until approximately stage VI of the seminiferous epithelium (Drabent *et al.*, 1996). Although expression of H1t has been extensively studied, its function remains unknown.

SUMMARY

This study has provided precise information about both the time that spermatocytes acquire meiotic competence and the biochemical differences in cell-cycle regulators between incompetent L/Z spermatocytes and competent pachytene spermatocytes. Competence to condense chiasmate bivalent chromosomes arises several days after spermatocyte chromosomes are fully synapsed and is temporally correlated with the first appearance of the CDC25C protein phosphatase and histone H1t in spermatocytes at mid-pachynema. This gives new information about the timing of events during meiotic prophase in mammalian spermatocytes and provides a context for the further study of recombination events in meiotic prophase and their temporal ordering. Additionally, correlations between acquisition of competence and the appearance of CDC25C and histone H1t provide the beginning of a list of candidate proteins required for regulation of the mammalian spermatocyte meiotic division. Nonetheless, the precise functional relationship between the progress of genetic and molecular events of recombination and the assembly of the cell-cycle machinery required for spermatocytes to get out of meiotic prophase and into metaphase is yet to be resolved. Determining if spermatocytes acquire a level of genetic competence, or completion of recombination, before assembling and localizing molecules required for chromosome condensation and segregation will be necessary in order to identify meiotic checkpoints and thus clarify mechanisms regulating onset of the meiotic divisions.

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